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Gene Expression Profiles in Mouse Liver Cells after Exposure to Different Types of Radiation

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Gene expression/Microarray/Mouse liver/Radiation quality/Endothelial cell/Kupffer cell.

The liver is one of the target organs of radiation-induced cancers by internal exposures. In order to elucidate radiation-induced liver cancers including Thorotrast, we present a new approach to investigate *in vivo* effects of internal exposure to α-particles. Adopting boron neutron capture, we separately irradiated Kupffer cells and endothelial cells in mouse liver *in vivo* and analyzed the changes in gene transcriptions by an oligonucleotide microarray. Differential expression was defined as more than 3-fold for up-regulation and less than 1/3 for under-regulation, compared with non-irradiated controls. Of 6,050 genes examined, 68 showed differential expression compared with non-irradiated mice. Real-time polymerase chain reaction validated the results of the microarray analysis. Exposure to α-particles and γ-rays produced different patterns of altered gene expression. Gene expression profiles revealed that the liver was in an inflammatory state characterized by up-regulation of positive acute phase protein genes, irrespective of the target cells exposed to radiation. In comparison with chemical and biological hepatotoxicants, inductions of Metallothionein 1 and Hemopexin, and suppressions of cytochrome P450s are characteristic of radiation exposure. Anti-inflammatory treatment could be helpful for the prevention and protection of radiation-induced hepatic injury.

INTRODUCTION

The biological effects of exposure to high linear energy transfer (LET) radiation have a particular relevance to radiation protection and risk assessment. Although internal exposure to high LET radiation is of a major concern, it is characterized by the existence of target organs and the difficulty of dose estimation. Thorotrast, a colloidal suspension of radioactive $^{232}ThO_2$ that naturally emits α-particles, was used as a radiographic contrast agent in the 1930s–1950s. More than half of intravasculary injected Thorotrast deposited in the liver caused liver cancers decades after the injection because of its life-long deposition and exposure to α-particles. Our histological examination of the liver from 144 cases of Thorotrast patients revealed intrahepatic cholangiocellular carcinoma (ICC, 25.7%), angiosarcoma (AS, 20.8%), hepatocellular carcinoma (HCC, 14.6%) and combined tumors (2.1%). Considering that Japan is an endemic area of hepatitis virus B and C, and that HCC comprises more than 80% of liver cancers, ICC and AS may be considered to be characteristic of Thorotrast-induced liver tumors. Our previous study showed that injected Thorotrast is phagocytosed by macrophages and radioactive Thorium is always migrating within the affected livers via Thorotrast-laden macrophages. These suggest that the liver is evenly exposed to α-particles at the organ level despite the short range of α-particles.[1)] Internal deposition of plutonium also causes chronic exposure to high levels of α-particles with increased risk of liver cancers including AS.[2)] Neither the deposited amount of Thorium nor the incubation period from injection to tumor induction is significantly different between cases with ICC and AS (manuscript in preparation). Consequently we thought that cell-to-cell interaction between irradiated macrophages and/or epithelial cells and parenchymal cells of the liver is involved in the development of ICC while direct irradiation of endothelial cells of the

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sinusoid is the principal contributor to the development of AS. A few studies also have been shown that exposure to α-particles induces liver tumors in mouse and other rodents.[3,4)]

Thermal neutrons cause the boron atom to split into an α-particle and a lithium nucleus via the boron neutron capture reaction (BNC). Both of these particles have a very short range (about one cellular diameter) and cause significant damage to the cell in which boron atoms are located. BNC therapy (BNCT) adopts this cytotoxic effect by selective delivery of boron-10 (^{10}B) to tumor cells: the short range nature of the effects of BNC minimizes the damage to adjacent normal cells. A large amount of ^{10}B compound can be administered in a liposome-incorporated form, which is then phagocytosed by macrophages. Conjugation of the liposome with polyethylene glycol (PEG) is known to increase blood levels of ^{10}B compounds and reduced uptake by macrophages.[5)] Recent radiological studies focus on the molecular mechanisms underlying transcriptional responses of mammalian cells to ionizing radiation. It is now apparent that the cellular reactions to ionizing radiation are complex and involve the activation of secondary messenger pathways and increased transcription of immediate early response genes.[6)]

These observations prompted us to adopt BNC to investigate *in vivo* effects of internal radiation exposure to α-particles. In this study, we prepared the ^{10}B-liposome treatment and the ^{10}B PEG-liposome treatment to expose Kupffer cells and endothelial cells to α-particles respectively, and analyzed the changes of gene expression using a microarray containing probes for 6,050 genes. As well as elucidation of the biological relevance of radiation, the present study also

Table 1. Primer sets for RT-PCR

Symbol	GenBank		Sequence	Size(bp)
AK3	AK005194	forward reverse	5'-GTG TGT TGG CCA AGA CTT TC-3' 5'-ATG TAT CCA GCG AGC AGT AAG-3'	236
Atp5b	AK010314	forward reverse	5'-GCA CAA TGC AGG AAA GGA TCA C-3' 5'-ACG TCA TAA TGC TCA TTG CCA AC-3'	241
ATP5c1	AK007063	forward reverse	5'-CGC CCC ATG GCA ACT CTG AAA G-3' 5'-GCC AAA GAA CCT GTC CCA TAC A-3'	160
Brap	AK013885	forward reverse	5'-AAA GGG CTG AAG TGC TGA ATC-3' 5'-TCT GGC GTT TGA CAG TAT CGG C-3'	211
Car3	AK003671	forward reverse	5'-CTT GAT GCC CTG GAC AAA AT-3' 5'-AGC TCA CAG TCA TGG GCT CT-3'	180
Egfr	AK004944	forward reverse	5'-TGA GCA ACA TGT CAA TGG ACT TAC-3' 5'-GCA TGT GGC CTC ATC TTG GAA C-3'	263
Galnt3	AK019995	forward reverse	5'-CAC TAT TTA CCC GGA AGC GTA TG-3' 5'-GTG GCA CGT GTA CAG AAT CAA TG-3'	139
Gsta3	AK014076	forward reverse	5'-TGA CCT GGC AAG GTT ACG AAG TG-3' 5'-CAT TAT CTC CAG ATC CGC CAC TC-3'	199
Hpxn	BB610094	forward reverse	5'-ATC TCA GCG AG GTG GAA GAA TC-3' 5'-CCT TCA CTC TGG CAC TCT CCA C-3'	215
Lcn2	AK002932	forward reverse	5'-CCA GTT CGC CAT GGT ATT TTT C-3' 5'-CAC ACT CAC CAC CCA TTC AGT T-3'	206
Mt1	AK018727	forward reverse	5'-ACC TCC TTG CAA GAA GAG CTG CT-3' 5'-GCT GGG TTG GTC CGA TAC TAT T-3'	160
Mt2	AK002567	forward reverse	5'-GGG TCC CCA CAT CTG TG TAA-3' 5'-CAA CGG CTT TTA TTG TCA GTT AC-3'	115
β-actin		forward reverse	5'-TTC TAC AAT GAG CTG CGT GTG G-3' 5'-GTG TTG GAA GGT CTC AAA CAT GAT-3'	110

contributes to the understanding of general idea of potential target molecules for cancer therapy.

MATERIALS AND METHODS

Mice and radiation

Male C3H/Hex mice (6 weeks old) were exposed to whole-body irradiation. For irradiation of mice by α-particles, specifically to macrophages and endothelial cells, ^{10}B-liposomes and polyethylene glycol (PEG)-^{10}B-liposomes were respectively administered. There were two mice analyzed by microarray, independently treated, and five mice by real time PCR. Mice used for these 2 assays were from 2 different courses of experiments. The ^{10}B compound sodium mercaptoundecahydrododecaborate (BSH) was used.[7] Each compound was suspended in physiological saline at a concentration of 4,000 ppm and 100 μl of ^{10}B-liposome solution and 300 μl of PEG-^{10}B-liposome solution were injected via the tail vein. Four hours (hrs) after the administration, the mice were exposed to neutron radiation at the Research Reactor Institute, Kyoto University (RRIKU). Before the irradiation experiments for gene expression, the neutron fluence was monitored by radioactivation of gold foils in the front and back of the mouse container. The average fluence of the thermal neutron source was 2.1×10^{12} n/cm^2 and the average flux was 2.3×10^9 n/cm^2/s at 5 MW. The boron concentration of the liver was measured by γ-ray spectrometry using a thermal neutron guide. We determined the exposure period at the calculated dose of 8.5 Gy at an organ level. For control irradiation, the mice were exposed to the neutron source for the same period as the BNC group. The contribution of neutrons and γ-rays to the total exposure was 4.2 cGy and 33 cGy, respectively. As a control for the quality of radiation, the mice were exposed to γ-rays at a dose of 8.5 Gy (0.34 Gy/min) with a ^{60}Co γ-ray source. Twenty hrs after irradiation, the mice were sacrificed by cervical dislocation. The dissected liver was immediately frozen and stored at –80°C until use. Animal experiments were approved by the Ethical Committee of the Institute of Development, Aging and Cancer, Tohoku University and were performed in accordance with institutional guidelines.

Oligonucletide microarrays

In accordance with 'Functional Annotation of Mouse' for the RIKEN full-length cDNA clone (http://fantom2.gsc.riken.go.jp/) and GenBank (http://www.ncbi.nih.gov/Genbank), 6,050 mouse genes were chosen for microarray analysis. These consisted of genes associated with signal transduction (766), cancer (506), autoimmune/inflammatory disease (455), cytokine/inflammatory response (267), stem cell

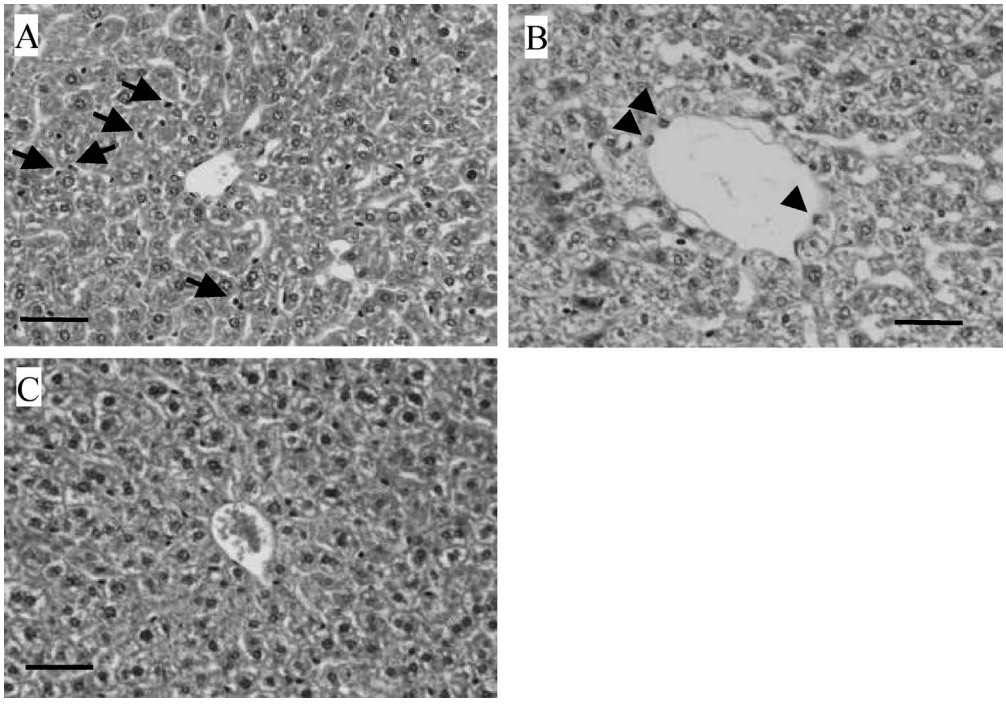

Fig. 1. Histological findings of the mouse liver following irradiation of Kupffer or endothelial cells. A: Compared with non-irradiated control, mice injected with ^{10}B-liposome solution showed a slight increase of the number and the size of Kupffer cells (arrows), indicating Kupffer cells were mainly irradiated (Kupffer exposure). B: Endothelial cells (arrow heads) were swollen in irradiated liver. The dilatation of sinusoids was noticed in mice injected with PEG-^{10}B-liposome, indicating sinusoidal endothelial cells were mainly insulted (Endothelial exposure). C: Non-irradiated control. Scale Bar: 50 μm.

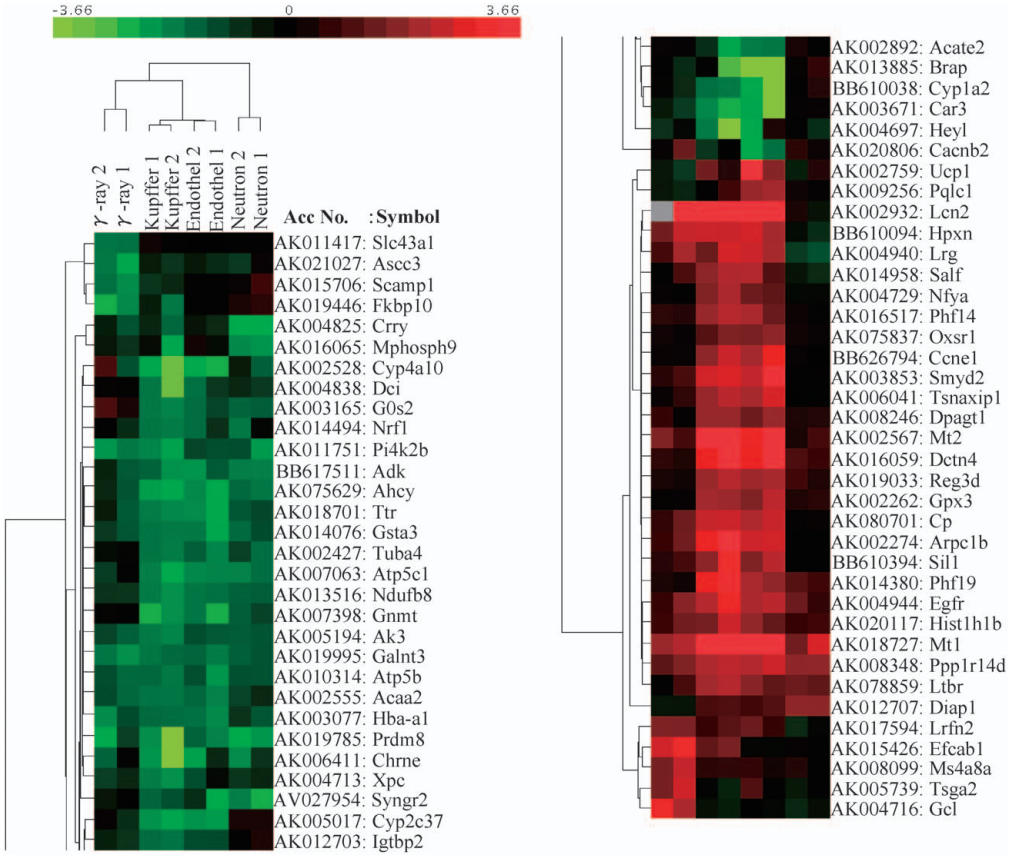

Fig. 2. Cluster analysis of individual mice according to the profile of gene expression examined. Mice from the same exposure group was the closest and the neutron exposure group was the most different from other groups.

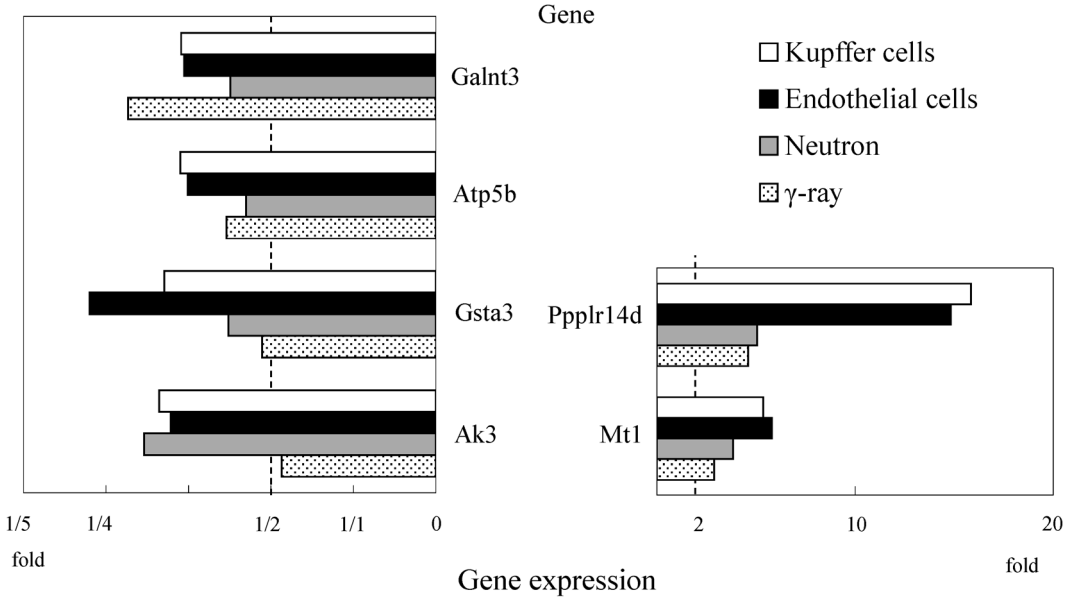

Fig. 3. Genes which are commonly over-expressed more than 2-fold or under-expressed less than a half following irradiation compared to control gene expression levels. Ak3: Adenylate kinase 3α, Gsta3: Glutathione S-transferase 3α, Atp5b: ATP synthase β subunit, Galnt3: UDP-N-acetyl-α-D-galactosamine-polypeptide, Mt1: Metallothionein 1, Ppp1r14d: Protein phosphatase 1, regulatory (inhibitor) subunit 14D.

(261), apoptosis (260), cardiovascular disease (240), neuroscience (197), toxicology/pharmacology (184), extracellular matrix/adhesion molecules (105), diabetes/obesity (105), developmental/regenerative disorder (102), cell cycle (99), and others (2,503).

After total RNA was extracted from the liver using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA), Poly-A RNA was separated using dT(25)-coupled magnetic beads (Dynal Biotech, Oslo, Norway). Individual mice were evaluated for the change in gene expression against pooled liver RNA

Table 2-A. Up and down-regulated genes in all the irradiated groups

	Gene	Symbol	GenBank	Function
Up	Metallothionein 1	Mt1	AK018727	Metal binding
	Protein phosphatase 1, regulatory (inhibitor) subunit 14D	Ppp1r14d	AK008348	Protein phosphatase inhibitor
Down	Adenylate kinase 3α	Ak3	AK005194	Adenine metaboilsm
	Glutathione S-transferase α3	Gsta3	AK014076	Detoxication
	ATP synthase β subunit	Atp5b	AK010314	ATP synthesis
	UDP-N-acetyl-α-D-galactosamine-polypeptide	Galnt3	AK019995	Secretion

Table 2-B. Commonly up and down-regulated genes by Kupffer cell specific and endothelial cell specific exposures to α-particles

	Gene	Symbol	GenBank	Function
Up	Lipocalin 2	Lcn2	AK002932	Anti-apoptosis
	Metallothionein 2	Mt2	AK002567	Metal binding
	Actin related protein 2/3 complex, subunit 1B	Arpc1b	AK002274	Cytoskeleton, protein trafficking
	Dynactin 4	Dctn4	AK016059	Cytoskeleton, protein trafficking
	PHD finger protein 19	Phf19	AK014380	Chromatin regulation
	Epidermal growth factor receptor	Egfr	AK004944	Cell growth
	SET and MYND domain containing 2	Smyd2	AK003853	Transcription
	Endoplasmic reticulum chaperone SIL1 homolog	Sil1-pening	BB610394	Molecular chaperon
	Hemopexin	Hpxn	BB610094	Metal transporter, antioxidant
	Ceruloplasmin	Cp	AK080701	Metal transporter
	Cyclin E1	Ccne1	BB626794	Cell cycle
	Translin-associated factor X (Tsnax) interacting protein 1	Tsnaxip1	AK006041	Cell cycle
	Regenerating islet-derived δ	Reg3d	AK019033	Cell growth
	Glutathione peroxidase 3	Gpx3	AK002262	Antioxidant
Down	PR domain containing 8	Prdm8	AK019785	Chromatin regulation
	CYP4A10	Cyp4a10	AK002528	Metabolism
	S-adenosylhomocysteine hydrolase	Ahcy	AK075629	Adenosine metabolism
	Glycine N-methyltransferase	Gnmt	AK007398	Methylation
	CYP2C37	Cyp2c37	AK005017	Metabolism
	Carbonic anhydrase 3	Car3	AK003671	Antioxidant
	ATP synthase γ subunit	Atp5c1	AK007063	ATP synthesis
	NADH dehydrogenase (ubiquinone) 1β subcomplex 8	Ndufb8	AK013516	Electron transport
	Transthyretin	Ttr	AK018701	Negative acute phase protein
	CYP1A2	Cyp1a2	BB610038	Metabolism

Table 2-C. Up and down-regulated genes by Kupffer cell specific exposure to α-particles

	Gene	Symbol	GenBank	Function
Up	Lymphotoxin B receptor	Ltbr	AK078859	Immunity
	Histone 1, H1b	Hist1h1b	AK020117	Chromosome organization
	PHD finger protein 14	Phf14	AK016517	Chromatin regulation
	Stoned B/TFIIA- α/β -like factor	Salf	AK014958	Transcription factor, membrane traficking
	Nuclear transcription factor-Y α	Nfya	AK004729	Transcription factor
	UDP-GlcNAc:dolichyl-phosphate N-acetylglucosamine photransferase 1	Dpagt1	AK008246	Protein glycosylation
Down	Nicotinic cholinergic receptor, ε polypeptide	Chrne	AK006411	Neurotransmitter/receptor
	Hairy/enhancer-of-split related with YRPW motif	Heyl	AK004697	Transcriptioin repressor
	3,2 trans-enoyl-CoA isomerase	Dci	AK004838	b-Oxidation of unsaturated fatty acids
	Xeroderma pigmentosum, complementation group C	Xpc	AK004713	DNA repair
	G0/G1 switch gene 2	G0s2	AK003165	G0/G1 transition
	Tubulin, α 4	Tuba4	AK002427	Cytoskeleton
	Nuclear respiratory factor 1	Nrf1	AK014494	Transcription factor
	Acetyl-CoA-acyl transferase 2	Acaa2	AK002555	Fatty acid oxidation
	Insulin-like growth factor binding protein 2	Igfbp2	AK012703	Cell growth regulation

Table 2-D. Up and down-regulated genes by endothel specific exposure to α-particles

	Gene	Symbol	GenBank	Function
Up	Uncoupling protein 1	Ucp1	AK002759	Heat production
	Leucine-rich α-2-glycoprotein1	Lrg1	AK004940	Acute phase protein
	PQ loop repeat containing 1	Pqlc1	AK009256	Electron carrier
	Oxidative-stress responsive 1	Oxsr1	AK075837	Cytoskeleton
Down	BRCA1 associated protein	Brap	AK013885	DNA repair
	Voltage-dependent Ca channel (β2)	Cacnb2	AK020806	Ion channel
	Hemoglobin α adult chain 1	Hba-a1	AK003077	Oxygen delivery
	Acyl-CoA thioesterase 2	Acate2	AK002892	Signal trans., protein traffick

Table 2-E. Up and down-regulated genes by γ-ray exposure

	Gene	Symbol	GenBank	Function
Up	EF hand calcium binding domain 1	Efcab1	AK015426	Ca binding
	Germ cell-less homolog	Gcl	AK004716	Differentiation
	Testis specific gene A2	Tsga2	AK005739	Testis specific
	Membrane-spanning 4-domains, subfamily A, member 8A	Ms4a8a	AK008099	Transporter
	Leucine rich repeat and fibronectin type III domain containing 2	Lrfn2	AK017594	Cell adhesion signal trans.
Down	FK506 binding protein 10	Fkbp10	AK019446	Immunosuppression
	Activating signal cointegrator 1 complex subunit	Ascc3	AK021027	Transcription coactivator
	Secretory carrier membrane protein 1	Scamp1	AK015706	Endocytosis
	Solute carrier family 43, member 1	Slc43a1	AK011417	Transporter

Table 2-F. Up and down-regulated genes by neutron exposure

	Gene	Symbol	GenBank	Function
Up	Diaphanous homolog 1	Diap1	AK012707	Cytoskeleton
Down	Complement receptor related protein	Crry	AK004825	Immunity
	Synaptogyrin 2	Syngr2	AV027954	Synaptic transmission
	Phosphatidylinositol 4-kinase type 2 β	Pi4k2b	AK011751	Inositol lipid biosyhthesis
	M-phase phosphoprotein 9	Mphosph9	AK016065	Cell cycle
	Adenosine kinase	Adk	BB617511	Signal transduction

from the five control non-irradiated mice. Complementary DNA (cDNA) probes were generated starting with 1 μg of polyA RNA using a CyScribe First-Strand cDNA Labelling kit (Amersham Biosciences Corp., Piscataway, NJ). cDNA from irradiated mice was labeled with Cy5 and that from control mice was labeled with Cy3. Pre-hybridization and hybridization were carried out on UltraGAPS coated slides in accordance with the manufacturer's manual (Corning, NY). The image was captured in a GenePix 4000B (Axon Instruments, Inc. CA). The quantification of gene expression arrays was performed by Array Vision software (Imaging Research Inc., Ontario, Canada). Cluster analysis of gene expression was performed by the War method.[8] The experiments were carried out in independent duplicates.

Real-time PCR

In order to validate the results from the microarray analysis we selected 12 genes (Table 1) and compared their gene expression as measured by microarray analysis and real-time PCR. DNaseI treated 500 ng of total RNA was used for the synthesis of cDNA using superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA corresponding to 10 ng of total RNA was amplified using Real-time PCR for the determination of gene expression using QuantiTect SYBR Green PCR Master Mix (QIAGEN K.K., Tokyo, Japan) in an iCycler (BIO-RAD, Hercules, CA). For the normalization of gene expression, a set of primers for β-actin was used.

RESULTS

Compared with non-irradiated controls, mice injected with ^{10}B-liposome solution showed a slight increase in the number and the size of Kupffer cells, indicating that Kupffer cells were irradiated (Kupffer exposure, Fig. 1A). The dilatation of sinusoids was noticed in mice injected with PEG-^{10}B-liposome, indicating sinusoidal endothelial cells were irradiated (Endothelial exposure, Fig. 1B). Liver tissues from mice exposed to γ-rays and neutrons revealed no remarkable histological changes (data not shown). In all cases, parenchymal hepatocytes did not show noticeable changes (Fig. 1).

Table 3. Comparison of gene expressioin levels between microarray and real-time PCR

Symbol	GenBank	endothelial			Kupffer			neutron			gamma		
		Real time-PCR	Micro-Array		Real time-PCR	Micro-Array		Real time-PCR	Micro-Array		Real time-PCR	Micro-Array	
		mean ± S.D.	Mouse 1	Mouse 2	mean ± S.D.	Mouse 1	Mouse 2	mean ± S.D.	Mouse 1	Mouse 2	mean ± S.D.	Mouse 1	Mouse 2
AK3	AK005194	0.66 ± 0.29	0.40	0.45	0.68 ± 0.10	0.29	0.27	0.84 ± 0.28	0.42	0.40	0.95 ± 0.33	0.38	0.48
Atp5b	AK010314	0.52 ± 0.06	0.22	0.44	0.72 ± 0.28	0.35	0.30	0.71 ± 0.17	0.43	0.44	0.90 ± 0.19	0.34	0.45
ATP5c1	AK007063	0.64 ± 0.06	0.27	0.26	0.68 ± 0.07	0.28	0.15	0.83 ± 0.29	0.33	0.28	1.47 ± 0.53	0.91	0.52
Brap	AK013885	0.69 ± 0.14	0.04	0.06	0.89 ± 0.32	0.94	0.13	1.23 ± 0.54	1.66	1.11	2.03 ± 0.71	0.62	0.97
Car3	AK003671	0.07 ± 0.053	0.04	0.20	0.14 ± 0.03	0.26	0.17	0.85 ± 0.27	1.14	1.09	0.53 ± 0.26	0.53	0.74
Egfr	AK004944	1.65 ± 0.84	5.41	4.90	3.39 ± 1.67	5.22	9.07	1.12 ± 0.69	1.68	2.81	1.93 ± 0.81	3.61	1.71
Galnt3	AK019995	0.19 ± 0.05	0.29	0.37	0.39 ± 0.20	0.30	0.34	0.68 ± 0.17	0.43	0.38	0.47 ± 0.06	0.24	0.30
Gsta3	AK014076	0.58 ± 0.14	0.18	0.30	0.85 ± 0.22	0.31	0.30	1.10 ± 0.21	0.46	0.34	0.67 ± 0.10	0.41	0.54
Hpxn	BB610094	36.53 ± 20.07	4.49	6.94	40.33 ± 17.23	6.42	6.38	2.56 ± 1.05	0.61	0.82	8.38 ± 5.3258	6.12	2.90
Lcn2	AK002932	1909.50 ± 558.5	94.79	95.38	1558.30 ± 133.6	34.90	62.41	1.94 ± 1.15	0.79	1.38	6.34 ± 5.20	128.21	–
Mt1	AK018727	24.22 ± 9.13	13.14	16.51	29.51 ± 8.88	15.35	16.38	1.67 ± 0.81	7.20	2.92	15.59 ± 10.66	5.12	4.11
Mt2	AK002567	38.46 ± 25.77	6.47	8.78	42.33 ± 7.71	12.84	19.36	2.57 ± 1.65	2.00	1.37	10.22 ± 7.43	1.99	3.32

Cluster analysis of the microarray results revealed that 2 mice from the same exposure group were the closest, indicating that all the experimental data in this study are reliable (Fig. 2). In this study, only the genes whose over-expression or under-expression compared with non-irradiated control levels which were consistently observed in 2 different mice with the same exposure levels were analyzed. In total, 161 genes were over-expressed by more than 2-fold and 32 genes

Table 4. Comparison of gene expression profiles between radiation exposures and hepatotoxicants

Gene	Symbol	GeneBank	Kupffer cells		Endothel		Neutrons		γ-rays		McMillian *et al*. (rat)	
			Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 1	Mouse 2	hepatotoxicants	PPA[a]
Macrophage activation/acute phase response												
Cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	BB610038	0.32	0.30	0.07	0.15	1.47	1.07	0.63	0.89	0.51	
CYP2C37	Cyp2c37	AK005017	0.23	0.20	0.21	0.23	1.35	1.36	0.60	1.11	0.63	
Fatty acid binding protein 5, epidermal	Fabp5	AK011551	2.85	1.64	1.28	1.76	1.17	1.46	0.71	0.45	2.75	
G-6-phosphatase, transport protein 1	G6pt1	AK003620	0.45	0.43	0.54	0.58	0.70	0.55	0.87	1.39	0.51	0.46
Hemopexin	Hpxn	BB610094	6.41	6.38	4.49	6.94	0.61	0.82	6.12	2.90	1.57	
Insulin-like growth factor binding protein, acid labile subunit	Igfals	AK004926	–	–	–	–	0.32	0.64	1.18	0.52	0.41	
Latent TGF-β binding protein 1	Ltbp1	AK020449	0.41	0.01	0.73	0.08	1.05	0.25	–	0.42	1.06	0.84
Metallothionein 1	Mt1	AK018727	15.35	16.38	13.14	16.51	7.20	2.92	5.12	4.11	1.44	0.23
Pyruvate kinase, muscle	Pkm2	AK002341	1.10	1.23	1.19	1.48	1.00	1.31	1.26	–	2.41	
Retinol binding protein 4, plasma	Rbp4	AK004839	0.32	0.34	0.24	0.40	0.63	0.58	0.61	0.73	0.66	
Superoxide dismutase 2	Sod2	AK002534	1.73	1.77	2.34	1.45	0.76	0.46	1.09	0.96	2.69	
Peroxisome proliferator												
Acetyl-CoA dehydrogenase, medium chain	Acadm	AK008149	0.62	–	0.81	0.87	0.76	0.88	0.52	0.50	0.47	
Brain acyl-CoA hydrolase	Bach	AK010646	1.23	1.25	1.26	1.45	1.06	1.26	0.88	1.02	0.82	2.00
3-hydroxybutyrate dehydrogenase	Bdh	AK009575	0.59	0.66	0.67	0.83	0.76	0.57	0.75	0.54	0.38	
CD36 antigen	Cd36	AK004192	1.20	1.09	1.17	1.15	0.93	1.30	1.10	0.69	1.01	2.87
Dodecenoyl-CoA delta isomerase	Dci	AK004838	0.30	0.10	0.55	0.32	0.54	0.65	0.89	1.10	0.37	1.73
2,4-dienoyl CoA reductase 1	Decr1	AK004725	1.22	1.10	1.11	1.10	1.14	1.22	1.00	0.95	0.45	
Epoxide hydrolase 2	Ephx2	AK002415	0.54	0.55	0.62	0.50	0.99	0.89	0.82	0.75	0.28	
Fatty acid CoA ligase, long chain 2	Facl2	AK004897	0.39	0.32	0.37	0.38	0.68	0.76	0.60	0.73	0.34	
3-hydroxy-3-methylglutaryl-CoA synthase 2	Hmgcs2	AK004865	0.46	0.38	0.48	0.34	1.01	1.21	0.69	1.40	0.44	
ER stress/chaperone protein /HSP												
Annexin A2	Anxa2	AK012563	0.75	1.26	1.45	1.38	1.14	0.93	1.66	–	2.36	
Calreticulin	Calr	AK075605	1.58	1.57	1.35	1.88	0.62	1.21	1.79	1.13	2.28	0.65
Protein disulfide isomerase-related	Pdir	AK012415	1.38	1.35	1.18	0.60	0.94	1.15	0.88	0.58	2.14	0.62
Metabolism												
S-adenosylhomocysteine hydrolase	Ahcy	AK075629	0.21	0.18	0.14	0.26	0.30	0.35	0.42	0.64	0.41	
Betaine-homocysteine methyltransferase	Bhmt	AK016283	1.49	1.70	1.27	1.52	1.20	1.39	1.58	1.99	0.24	
Sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	AK002700	0.41	0.43	0.35	0.49	0.57	0.59	0.92	0.99	0.43	
Other functions												
G0/G1 switch gene 2	G0s2	AK003165	0.30	0.27	0.48	0.34	0.38	0.36	1.35	2.07	0.60	
Kininogen	Kng	AK005547	1.71	2.65	2.55	2.70	1.37	1.21	1.61	1.04	1.16	0.68
Solute carrier family 34 , member 2	Slc34a2	AK004832	0.45	0.23	0.81	1.78	0.74	1.18	1.24	1.37	1.06	1.59

Light gray background: Under-expressed genes; Black: Over -expressed genes
[a]: Peroxisome proliferator agonist

were over-expressed by more than 3-fold by exposure to either type of radiation. Of the under-expressed genes, 194 showed an expression level of less than 1/2, compared to the control levels and 36 genes showed less than 1/3 compared to the control levels. Approximately the same number of genes showed over-expression of between 2- and 3-fold or under-expression of between a half and a third. Therefore, we defined a gene to be up-regulated when the mean value of its expression levels in exposed mice showed more than a 3-fold over-expression, and down-regulated if its expression level was less than 1/3 compared with non-irradiated mice.

In terms of gene expression profile, Kupffer and endothelial exposures were the most similar to and the neutron exposure group was the most different from other groups (Fig. 2). For commonly up- and down-regulated genes in all the exposure groups, we picked up genes with a level of over-expression of more than 2-fold and under-expression of less than a half among all the radiation groups. Commonly up-regulated genes were metallothionein 1 (Mt1) and protein phosphatase 1, regulatory (inhibitory) subunit 14D (Ppp1r14d). Commonly down-regulated genes were adenylate kinase 3α (Ak3), glutathione S-transferase 3α (Gsta3), ATP (Adenosine triphosphate) synthase β subunit (Atp5b) and UDP (uridine diphosphate)-N-acetyl-α-D-galactosamine-polypeptide (Galnt3) (Fig. 3 and Table 2-A). Commonly up- and down-regulated genes between Kupffer cell irradiation and endothelial cell irradiation are shown in Table 2-B. There were 14 up-regulated genes: 5 associated with cell-cycle regulation, 3 associated with intracellular transportation, 3 that code for metal binding proteins and 3 others. There were 10 down-regulated genes, composed of 3 cytochrome P450 (CYP) genes, 3 associated with ATP synthesis and 4 others. For the genes whose changes in expression were specific to irradiated Kupffer cells, molecules associated with transcription including histone H1 were 3 of 6 up-regulated genes. Of the 9 down-regulated genes, 2 each were respectively associated with cell cycle, transcription and fatty acid metabolism, and 1 was involved in DNA repair (Table 2-C). Among genes specific to endothelial exposure, acute phase protein and cytoskeleton associated gene were up-regulated. Down-regulated genes were associated with signal transduction, protein trafficking and DNA repair (Table 2-D). In contrast to cell specific exposure groups, the genes with altered expression by neutrons or γ-rays were small in number and did not appear to possess significantly different characteristics (Table 2-E and -F). In each Table, up-regulated genes are presented in decreasing order and down-regulated genes in increasing order. All primary Microarray data are available at the site of GEO (http://www.ncbi.nlm.nih.gov/project/geo/) (data No. GSE9290).

In order to validate the consistency of microarray analysis in the present study, we compared gene expression levels of selected genes between microarray and real-time PCR. We determined the mean value of expression of the selected genes in 5 independent mice from each exposure group. This was compared with those in pooled RNA from 5 non-irradiated mice. The qualitative changes in gene expression levels were consistent between these analyses. However, the quantitative difference was greater in real-time PCR than in microarray analysis, both in up- and down-regulated genes (Table 3).

Since radiation exposure could be hepatotoxic, we compared the results of our present study with the results by McMillian *et al*.[9] They performed microarray analysis of gene expression in rat liver 24 hrs after administration of various kinds of hepatotoxic compounds. We picked up genes whose expression level increased more than 2-fold or decreased to less than 1/2 of the control level in their or our study (Table 4). Over-expression of Mt1 and hemopexin (Hpxn), and under-expression of CYP were prominent in radiation-exposed samples compared with those undergoing administration of hepatotoxic chemical compounds and peroxisome proliferator agonists.

DISCUSSION

Gene array analysis of RNA from irradiated tissues is an effective tool for identifying genes of potential interest in the development of tissue injury. Since Thorotrast naturally emits α-particles and causes liver cancers, evaluating changes in gene expression in the liver irradiated with α-particles might help us to understand how Thorotrasts induce liver cancer. In order to analyze the effect of target cell specificity and quality of irradiation on gene expression in the liver, we intended to separately expose Kupffer and endothelial cells to α-particles using BNC, and performed oligonucleotide microarray analysis. Ishida *et al*. reported that 4 hrs after injection into mice, 5% of bare liposomes and 50% of PEG-liposomes are retained in the blood, respectively, whereas, 70% of bare liposomes and 15% of PEG-liposomes accumulate in the liver, respectively.[10] Assuming that liposomes in either form are phagocytosed by Kupffer cells in the liver, the dose ratio of Kupffer group to endothelial group is 4.7 folds in Kupffer cell group and 1/10 in endothelial cell group in this study. Although we could not completely separate target cells for α-particle exposure, we think these numbers were satisfactory because of internal exposure experiments of the mouse. The cellular responses of Kupffer and endothelial groups were the closest to other groups, whilst the group exposed to neutrons showed greatest variations from other groups (Fig. 2). This suggests that cellular responses are mainly determined by the quality of radiation, that is, dependent on exposure to high LET particles or low LET photons.

Acute phase response refers to changes in concentrations of a number of plasma proteins, termed acute-phase proteins (APPs) which reflect re-orchestration of the pattern of gene

expression in hepatocytes in response to a variety of systemic injuries. An APP has been defined as one whose plasma concentration increases (positive APP) or decreases (negative APP) by at least 25% after injury. In the present study, we detected significant changes of the level of APPs such as Hpxn, ceruloplasmin (Cp) and transthyretin (Ttr) commonly in Kupffer and endothelial exposures (Table 2-B). These indicate that the alterations of gene expression in this study reflect those of hepatocytes even after Kupffer cells and endothelial cells were specifically exposed to α-particles. Cp has a scavenger activity[11] and Hpxn acts as an antioxidant by its strong heme binding and iron homeostasis properties.[12] During inflammation, macrophages and endothelial cells secrete the so-called pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), Interleukin-1β (IL1β) and IL6.[13] Mt also has antioxidant activity and this gene expression is induced by IL6.[14] Lipocalin is also an APP involved in a mammalian defense mechanism against bacterial infection and works by binding to the iron group within bacterial iron-containing siderophores.[15] Interestingly, acute lung injury in mice induced by lipopolysaccharide and diesel exhaust[16] particles up-regulates lipocalin 2 and Mt2 gene expressions.[16] The present study suggests that pro-inflammatory cytokines are secreted by irradiated macrophages and endothelial cells, especially those exposed to α-particles. Mouse macrophages are activated after whole body irradiation to 4 Gy of γ-rays. However, this activation is not a direct effect of radiation but an indirect effect induced by phagocytosis of apoptotic cells after irradiation.[17] In the present study, the destruction of macrophages and endothelial cells in the spleen was also observed (data not shown). We need to take account of the indirect effects on the spleen of radiation exposure when considering liver carcinogenesis of Thorotrast patients, because the spleen in Thorotrast patients is drastically reduced in size compared to the liver. The changes in gene expression profile commonly observed after Kupffer cell and endothelial cell exposures revealed that hepatocytes are in the state of inflammation and are tending towards proliferation at the cost of metabolic activities. Hepatocytes also actively perform quality control of substances by up-regulation of intracellular protein trafficking.

Expression of genes encoding molecules associated with transcription was up-regulated and expression for those associated with signal transduction was down-regulated in the liver. Further study to characterize molecules involved in these gene expressions would elucidate radiation carcinogenesis, especially that of Thorotrast-induced liver tumors. It is noticeable that *epidermal growth factor receptor* (*EGFR*) and *cyclin E1* gene expressions were up-regulated in the liver whose Kupffer cells or endothelial cells were exposed to α-particles, whereas *xeroderma pigmentosum, complementation group C (XPC)* and *insulin-like growth factor binding protein 2 (IGFBP2)* gene expressions were down-regulated in Kupffer cell irradiated group and *BRAP* gene expression in endothelial cell irradiated group. The level of *EGFR* gene expression in tumors has been correlated to the degree of radiation resistance.[18] Exposure of the breast cancer cell line, MCF-7 to γ-rays enhanced EGFR gene expression concomitant with overexpression of its ligand, TGFα,[19] resulting in enhanced cell growth by irradiation.[20] Recently, new targets for cancer treatment have been identified in head and neck squamous cell carcinomas (HNSCC) as playing key roles in tumor proliferation and metastasis. The first one led to the approval of a molecularly based therapy in HNSCC is EGFR.[21] Cyclin E initiates cells to pass from G1- to S-phase and controls genomic stability. High level expression of cyclin E has been associated with the initiation or progression of various human cancers.[22] Transgenic mice in which cyclin E is constitutively expressed develop malignant diseases, supporting the notion of cyclin E as a dominant onco-protein.[23] XPC carries out the first step of global genome repair in nucleotide excision repair. The lack of the XPC protein is associated with UV-induced skin tumors but not with hypersensitivity against ionizing radiation.[24] IGFBP2 in breast cancer cell lines is a marker of resistance against anti-estrogen therapy.[25] It has also been shown that IGFBP2 plays a key role in the activation of the Akt pathway and collaborates with K-Ras or platelet-derived growth factor beta polypeptide (PDGFB) in the development and progression of two major types of glioma.[26] These results suggest that irradiated liver is in the condition toward cancer induction.

Comparison with the data of McMillian *et al.*[9] revealed that all types of radiation exposure investigated involve macrophage activation rather than peroxisome proliferation (Table 4). Up-regulations of Mt1 and Hpxn, and down-regulation of CYP and retinol binding protein 4 (Rbp4) are characteristic of radiation exposure. Steatohepatitis including alcoholic fatty liver is well known to be a precursor status toward liver fibrosis and liver cancer. Since diverse causes of steatohepatitis are characterized by increased mitochondrial (mt) reactive oxygen species (ROS) production, limited repair of mtDNA and accumulation of oxidatively damaged DNA,[27] cellular reaction against radiation toward lipogenesis may indirectly contribute to DNA insult by high LET radiation. Therefore, intensive or preventive anti-inflammatory treatment could help radiation-induced injury. Most of the genes involved in ATP synthesis, oxidative phosphorylation, copper ion homeostasis and electron transport were induced by both continuous and acute exposure of *Saccharomyces cerevisiae* to γ-rays.[28] The results were concordant with the present study though we focused on *in vivo* radiation of the mouse liver. Furthermore, it has been shown that microvascular endothelial cells are the primary target to initiate intestinal radiation damage.[29] These similarities indicate that cell-to-cell interaction in response to radiation *in vivo* is the result of amplification of *in vitro*

signals. In order to separate the effects of irradiation on parenchymal, Kupffere and endothelial cells, experiments involving irradiation of these cell types after cell fractionation are underway in our laboratory.

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